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Thiamine Pyrophosphate Riboswitches Are Targets for the Antimicrobial Compound Pyrithiamine

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Summary

Thiamine metabolism genes are regulated in numerous bacteria by a riboswitch class that binds the coenzyme thiamine pyrophosphate (TPP). We demonstrate that the antimicrobial action of the thiamine analog pyrithiamine (PT) is mediated by interaction with TPP riboswitches in bacteria and fungi. For example, pyrithiamine pyrophosphate (PTPP) binds the TPP riboswitch controlling the *tenA* operon in *Bacillus subtilis*. Expression of a TPP riboswitch-regulated reporter gene is reduced in transgenic *B. subtilis* or *Escherichia coli* when grown in the presence of thiamine or PT, while mutant riboswitches in these organisms are unresponsive to these ligands. Bacteria selected for PT resistance bear specific mutations that disrupt ligand binding to TPP riboswitches and derepress certain TPP metabolic genes. Our findings demonstrate that riboswitches can serve as antimicrobial drug targets and expand our understanding of thiamine metabolism in bacteria.

Introduction

Riboswitches are conserved RNA elements that form receptors for specific small molecules and regulate gene expression in response to ligand binding [1–3]. For all classes of riboswitches studied so far, ligand binding by these RNAs can occur in the complete absence of protein. Riboswitch-regulated genes are involved in numerous bacterial biosynthetic pathways, and more than 2% of the genes in certain bacteria are riboswitch regulated [4]. Riboswitches typically exhibit remarkable specificity, 100- to 1000-fold or more, for their cognate ligands versus highly related compounds [5–7]. This high level of molecular discrimination allows these RNA genetic switches to selectively control gene expression even in the presence of many chemically related compounds in cells.

A class of riboswitches that binds thiamine pyrophosphate (TPP) regulates genes that code for protein products involved in the biosynthesis and transport of thiamine in numerous bacteria. While most bacteria can synthesize thiamine de novo, all animals and many fungi require supplemental thiamine or its immediate precursors [8, 9]. Intracellular thiamine is then phosphorylated to TPP and participates as a coenzyme for enzymes that cleave carbon-carbon bonds adjacent to a carbonyl group for processes including glycolysis, the citric acid cycle, and the pentose phosphate pathway. Maintaining adequate levels of TPP and sufficient activity of TPP-utilizing enzymes is essential to all known cellular life forms.

Decades ago, when vitamins were first being identified and structurally characterized, pyrithiamine (PT) was designed to be an isosteric pyridine analog of thiamine and synthesized for the study of thiamine metabolism (Figure 1A) [8–10]. PT was quickly shown to be toxic in small amounts to fungi and bacteria that require supplemental thiamine [11, 12] and to induce thiamine deficiency in mice [13] and pigeons [14]. It is now known that PT, like thiamine, is phosphorylated in cells to pyrithiamine pyrophosphate (PTPP), most likely by thiamine pyrophosphorylase (TPK) [8, 15, 16]. In eukaryotes, PT inhibits the conversion of dietary thiamine to TPP and PTPP inhibits enzymes that use TPP as a coenzyme [15, 17–20]. In mammals, PT crosses the blood-brain barrier, inhibits TPK, reduces thiamine transport into the brain, and leads to an overall reduction in total thiamine levels [21]. These features are routinely exploited to induce thiamine deficiency in animal models to study thiamine deficiency disorders (e.g., [22–24]). Thus, PT and PTPP compete for thiamine and TPP binding sites on proteins. Bacteria and fungi that synthesize TPP de novo might be expected to overcome the effects of PT by upregulating thiamine production. However, these organisms are also susceptible to the effects of PT, and for several decades, the full spectrum of toxic effects of this thiamine analog on bacteria and fungi has remained unknown [25, 26].

Intriguingly, PT had previously been shown to alter the regulation of thiamine biosynthesis enzymes in bacteria and fungi. In *E. coli*, for example, PT-resistant mutants show derepression of enzyme activities leading to biosynthesis of thiamine and TPP [26]. In the filamentous fungus *Aspergillus oryzae*, transcripts of a putative thiamine precursor biosynthetic gene, *thiA*, are reduced in number in cells grown with either thiamine or PT. A resistant mutant has been obtained that shows derepression of the *thiA* gene with both compounds [25]. Interestingly, this resistance is conferred by a point mutation in the 5′ untranslated region (5′-UTR) of the *thiA* transcript, while no changes occur in the protein coding region. The 5′-UTR is predicted to contain two introns; deletions in the 5′-UTR alter thiamine-mediated regulation and also cause aberrant splicing [27]. This suggested a role for PT in altering proper processing of the *thiA* 5′-UTR and

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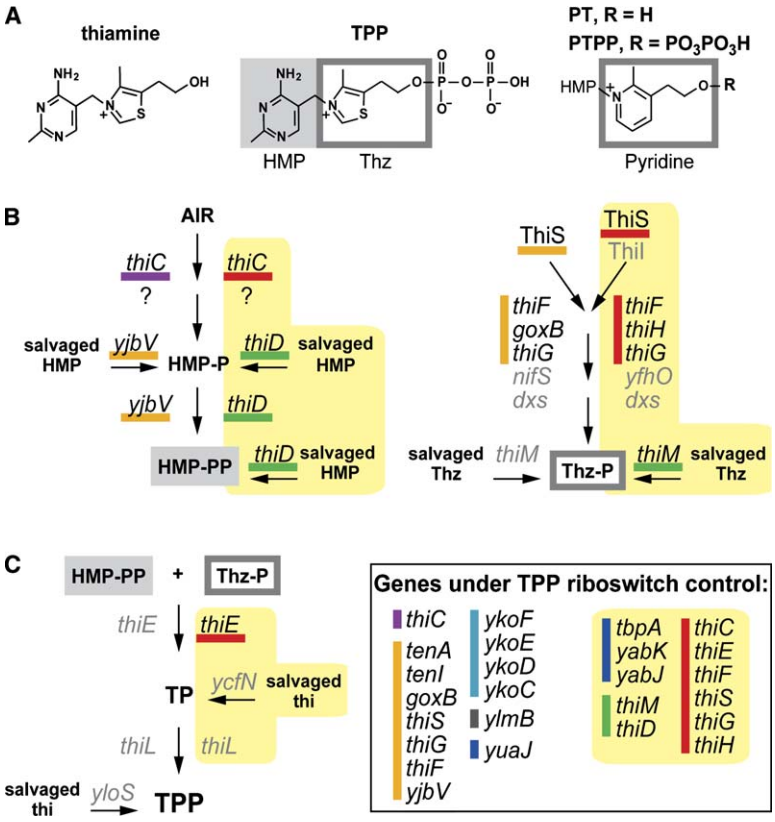

Figure 1. Structure and Biosynthesis of TPP

(A) Chemical structures of thiamine, TPP, PT, and PTPP. PT differs as shown with the replacement of the thiazole (Thz) moiety with a pyridine ring (boxed). The hydroxymethylpyrimidine (HMP) moiety (shaded) is unchanged from thiamine in both PT and PTPP.

(B) The biosynthesis of HMP-PP and Thz-P in *B. subtilis* and *E. coli*. Genes regulated by TPP riboswitches in *B. subtilis* are shown to the left of the reaction pathway and in *E. coli* to the right of each pathway and shaded yellow. See Table S1 for additional information. Black type: genes regulated by TPP riboswitches; gray type: genes not regulated by TPP riboswitches. Colored bars denote transcriptional units. AIR, 5-aminoimidazole ribonucleotide; HMP-P, hydroxymethylpyrimidine monophosphate; HMP-PP, hydroxymethylpyrimidine pyrophosphate; Thz-P, thiazole monophosphate. In *E. coli*, the activity of ThiC requires at least one other cellular factor [36], so it is possible that *B. subtilis* requires an additional factor as well, denoted with question marks.

(C) The biosynthesis of TPP from HMP-PP and Thz-P. Notations are as in (B). TP, thiamine monophosphate. Box: complete list of genes controlled by TPP riboswitches in *B. subtilis* (left) and *E. coli* (right, shaded yellow).

led us to investigate the possibility that PT interacts with riboswitches in the 5′-UTRs of both bacteria and fungi.

A precedent for interaction between an antimicrobial compound and a riboswitch exists. Previously, we reported that a toxic analog of lysine, *S*-(2-aminoethyl)-L-cysteine (AEC), binds to the lysine riboswitch with only a 30-fold reduction in affinity in vitro compared to lysine [28]. Elevated AEC concentrations regulate expression of a reporter gene that carries a lysine riboswitch. Point mutations of conserved nucleotides in the lysine riboswitch confer resistance to AEC toxicity and also abolish regulation of reporter gene expression. In light of these results, we examined the interaction of TPP riboswitches from fungi and bacteria with both PT and PTPP. As a result of the observed pattern of riboswitch binding and gene regulation induced by PT and its derivatives, we conclude that binding of PTPP to TPP riboswitches contributes to the antimicrobial effect of this compound. We also provide evidence that spontaneous bacterial resistance to PT emerges through mutations to a TPP riboswitch controlling key thiamine metabolic genes. These findings have implications both for practical development of new antibacterial drugs and for the basic understanding of coenzyme biosynthesis and metabolism.

Results and Discussion

TPP Riboswitches Regulate Bacterial Thiamine Biosynthesis and Transport

Both *E. coli* and *B. subtilis* can either synthesize TPP or import and phosphorylate thiamine, and both are normally susceptible to the toxic effects of PT (see Figure S1 in the Supplemental Data available with this article online). There are three operons in *E. coli* that were determined using bioinformatics to each carry a conserved TPP riboswitch motif in the 5′-UTR, as listed in the RNA family database Rfam [29, 30]. Expression of these genes is known to be attenuated in response to TPP in certain bacteria [31]. We found that two of these RNAs exhibit protein-free binding of TPP in vitro and that these elements confer genetic regulation to reporter genes in vivo [6]. In *B. subtilis*, there are five transcriptional units (two operons and three single genes) that carry TPP riboswitch motifs [32]. These are all predicted to form intrinsic transcription terminators in the presence of TPP and to repress expression of thiamine biosynthetic and transport genes. One operon has been confirmed to undergo transcriptional attenuation with TPP [33]. Additional information on these genes and their functions are listed in Table S1.

The TPP riboswitch motifs of *E. coli* and *B. subtilis* are located upstream of open reading frames (ORFs) for numerous thiamine metabolism proteins. Thiamine is synthesized by an enzymatic condensation of two precursors that provide the pyrimidine moiety (HMP) and thiazole moiety (Thz) of thiamine [34, 35]. The precursors are either synthesized de novo, transported, or salvaged and phosphorylated to HMP-PP and Thz-P prior to the condensation. Nearly all of the genes needed for HMP-PP and Thz-P synthesis are located downstream of TPP riboswitch motifs (Figure 1B). The details of HMP-PP synthesis are not completely understood [36], but TPP riboswitches are associated with genes that are critical for HMP-PP synthesis and phosphorylation. Thz-P synthesis has been completely reconstituted with both

E. coli and *B. subtilis* proteins in vitro, and this pathway involves several steps and numerous gene products [37, 38]. The gene products of *nifS*, *yfhO* (also known as *iscS*), and *dxs* are needed both for thiamine metabolism and for other pathways, so it is not surprising that they are independent of TPP riboswitch control. Some differences in thiamine synthesis exist between *E. coli* and *B. subtilis* and are detailed in Figure 1. For example, phosphorylation of salvaged Thz is riboswitch associated in *B. subtilis* but not in *E. coli*.

The expression of proteins involved in the final synthetic steps of TPP is not extensively regulated by TPP riboswitches. The precursors HMP-PP and Thz-P are condensed to thiamine monophosphate (TP) by ThiE, with the pyrophosphate of HMP-PP serving as the leaving group for the reaction and the phosphate of Thz-P being retained in TP (Figure 1C). The *thiE* gene is associated with a TPP riboswitch in *E. coli* but not *B. subtilis*. Both newly synthesized TP and salvaged thiamine must be phosphorylated to TPP. None of the kinases for these phosphorylations are associated with TPP riboswitches.

Not shown in Figure 1 are pathways for transport of thiamine compounds. Although thiamine transport has not been fully characterized in these organisms, some details are known. In *E. coli*, the riboswitch-regulated operon *tpbA-yabK-yabJ* encodes proteins that transport thiamine, TP, and TPP [35]. In *B. subtilis*, the *ykoF-C* operon and *yuaJ* putatively code for transport proteins for HMP and thiamine, respectively [39]. The levels of these proteins also appear to be controlled by TPP riboswitches. PT is known to be imported into yeast cells via thiamine transport proteins [18]. Therefore, PT could enter bacterial cells by analogous transporters in addition, perhaps, to passive diffusion.

The presence of TPP riboswitch motifs in the mRNAs of thiamine metabolic genes suggests a feedback loop is operating whereby TPP levels control the import of thiamine and the synthesis of thiamine precursors. Free TPP in the cell should bind simultaneously to all TPP riboswitches in actively transcribed mRNAs in these pathways and globally influence expression of these genes. If a thiamine analog such as PT could bind these riboswitches in vivo, a similar widespread reduction in expression of thiamine metabolic genes should occur. To begin our assessment of whether PT toxicity could be due to interactions with TPP riboswitches, we first investigated the binding behavior of TPP riboswitches in vitro with thiamine, PT, and their pyrophosphorylated derivatives.

Bacterial TPP Riboswitches Bind PTPP

The majority of known thiamine biosynthesis genes in *B. subtilis* are contained in the *tenA-tenI-goxB-thiS-thiG-thiF-yjbV* operon (called *tenA* hereafter). To complement the published data that TPP attenuates transcription of this operon in vitro [33], we tested direct binding of TPP to the 5′-UTR of the *tenA* operon (Figure 2). The construct employed for these tests included both the aptamer for binding TPP (Figure 2B) and the expression platform for transducing ligand binding into gene expression regulation [40]. The expression platform in this case controls the formation of an intrinsic terminator of transcription (Figure 2A). As for many riboswitch classes, ligand binding is permitted when the base-paired structure termed P1 is formed. The intrinsic terminator then forms and transcription of the downstream genes is repressed. If levels of TPP are low and no ligand is bound to the riboswitch, the nucleotides in P1 are free to form an antiterminator and transcription of several thiamine biosynthetic genes in the operon proceeds.

An RNA corresponding to nucleotides −185 to −23 relative to the translation start site of *tenA* was transcribed and submitted to an in-line probing assay where changes in RNA structure due to ligand binding can be determined by changes in the levels of spontaneous RNA degradation [41–43]. TPP was incubated with the RNA in a range of concentrations from 1 nM to 100 μM. The results confirmed the *tenA* RNA is a functional TPP aptamer (Figure 2C). As the concentration of TPP was increased, significant changes in spontaneous cleavage occurred at nucleotides such as A36, A61, and A63. The apparent K_D of the riboswitch aptamer for TPP was determined by in-line probing as described previously [6] and found to be 50 nM (Table 1). To determine whether TPP riboswitches are targets of pyrithiamine, the PT-resistant strain 1A481 was obtained from the *Bacillus* Genetic Stock Center (The Ohio State University) and sequenced at each of the five *B. subtilis* TPP riboswitches. A single nucleotide change from G to A in the *tenA* 5′-UTR was found (Figure 2A). In-line probing of the mutant riboswitch revealed much less pronounced changes in the pattern of spontaneous degradation including at sites A36, A61, and A63 (Figure 2D) with a reduction in affinity by 36-fold to a K_D of 1.8 μM (Table 1).

High (10 μM) concentrations of either thiamine or PT showed little evidence of binding to the wild-type riboswitch (Figure 2C). The affinities for either ligand were found to be 100- to 1000-fold poorer than for TPP (Table 1). This loss in affinity for the nonphosphorylated form is in good agreement with previous results for thiamine and TPP with *E. coli* riboswitches [6]. To mimic the natural ligand more closely in the in-line probing assay, PTPP was synthesized from PT and phosphoric acid (see Experimental Procedures) and tested for binding to the wild-type and mutant riboswitch constructs. The wild-type *tenA* riboswitch shows a TPP-like pattern of spontaneous RNA degradation in the presence of a modest 100 nM concentration of PTPP (Figure 2C). The mutant *tenA* riboswitch fails to show TPP-like structural modulation even with significantly higher (1 μM) PTPP. The affinities for binding PTPP by the wild-type *tenA* or the mutant riboswitch constructs are nearly identical to those for binding TPP (Table 1). The difference in affinity between PT and PTPP matches the difference in affinity between thiamine and TPP, suggesting that these analogs might be bound by TPP riboswitches using the same molecular contacts. Therefore, PT in the form of PTPP is very likely to interact with TPP riboswitches in vivo.

The mutant riboswitch from the PT-resistant *B. subtilis* strain has an ~40-fold reduced affinity for TPP and a 10-fold reduced affinity for PTPP. The G to A mutation at nucleotide 94 is a good candidate to confer PT resistance by disrupting proper TPP aptamer formation in the riboswitch. P1 is weakened by the mutation but the antiterminator is not expected to be adversely affected (see Figure 2A legend). The same trend is also observed in

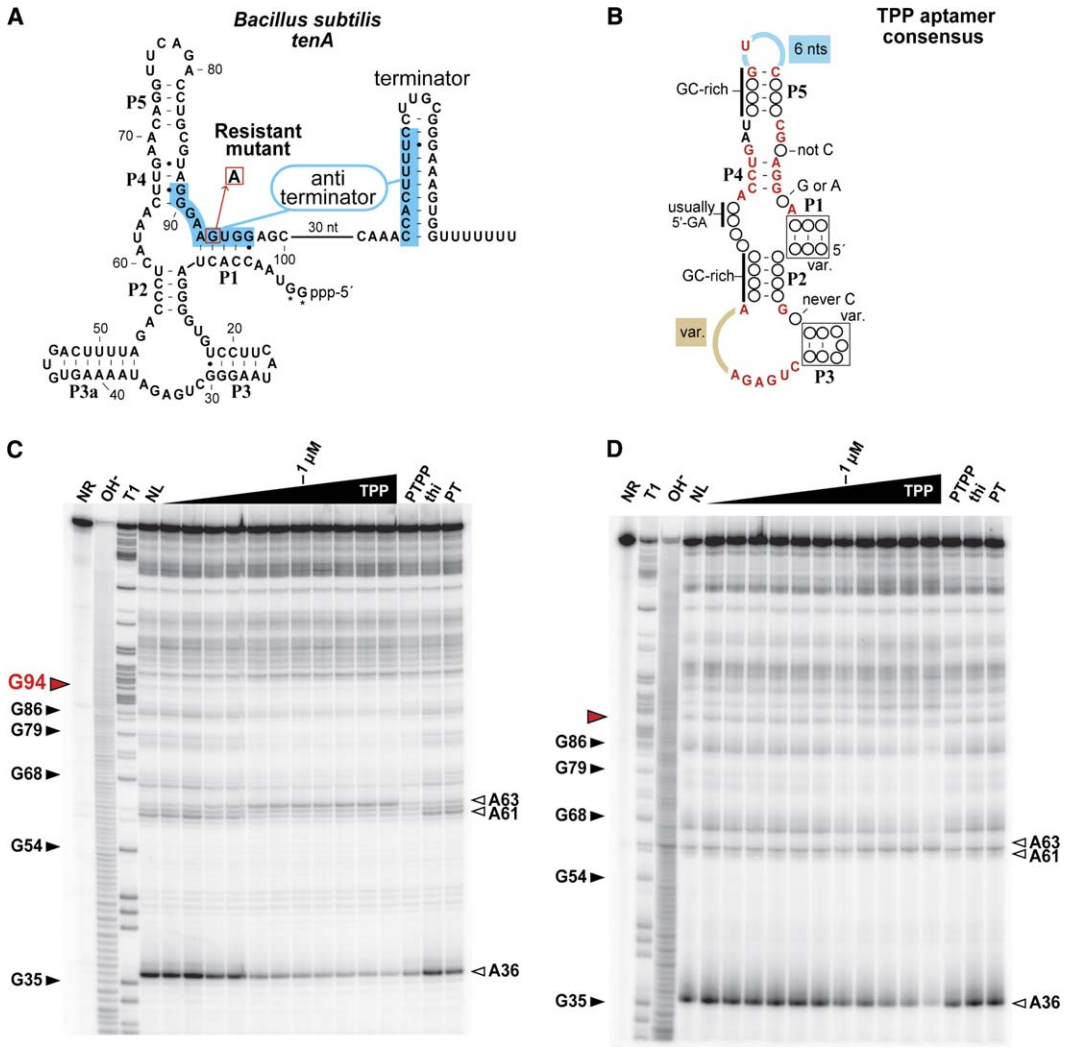

Figure 2. Consensus and *B. subtilis tenA* TPP Riboswitches

(A) The *B. subtilis tenA* 5′-UTR constructs used for in-line probing. Depicted are nucleotides −185 to −23 (relative to the translation start) of the wild-type 5′-UTR. The resistant mutant sequence is derived from the PT-resistant strain 1A481 and bears the mutation shown. The sequence 5′-GCGUAGGGAA AUGGAGCCAA ACCACUUUUC CUUGCGGG-3′ from this mutant has a change in free energy on folding of −14.4 kcal/mol as calculated on the mfold server [62, 63]. P1–P5 denote base-paired elements. G residues denoted with asterisks were added to aid in vitro transcription.

(B) Consensus TPP riboswitch, reprinted with permission from the journal RNA. Red nucleotides are conserved in >90% of 100 bacterial and archaeal TPP riboswitches. Circles denote nucleotides whose exact identity is not conserved. var., variable regions.

(C) In-line probing of the wild-type *tenA* riboswitch. RNA was incubated with concentrations of TPP ranging from 1 nM to 100 μM, or with PTPP at 100 nM, thiamine (thi) at 10 μM, or PT at 10 μM. Markers were generated by partial digestion with RNase T1 to cleave after G residues (e.g., those labeled at left in figure) or with alkali, denoted OH^- NR and NL indicate no reaction and no ligand, respectively. Sites of significant ligand-induced structural modulation are labeled A36, A61, and A63. The red arrowhead indicates the expected position of the mutation G94A shown in (D).

(D) In-line probing of the mutant *tenA* riboswitch. RNA was incubated with ligands as in (C), except with PTPP raised to 1 μM. Other notations are as in (C).

TPP/PTPP binding with the *thiC* operon riboswitch from *E. coli* (data not shown). The wild-type *E. coli* riboswitch binds TPP and PTPP well but not thiamine or PT. The PT-resistant *E. coli* strain PT-R1 had been created many years ago [44] to investigate thiamine metabolism, and the mutation that conferred PT resistance was mapped at that time to the *thiC* operon region. We obtained the strain from the *E. coli* Genetic Stock Center (Yale University), sequenced the 5′-UTR of the *thiC* operon, and found a deletion of the conserved G between P2 and P3 (Figure 2B). As predicted due to the disruption of the consensus aptamer, this mutant riboswitch does not bind either TPP or PTPP well.

These results indicate that bacterial TPP riboswitches bind PTPP well in vitro and that PT resistance is associated with reduced ligand binding by mutated TPP riboswitches in bacteria. We next investigated whether the specificity of a eukaryote TPP riboswitch would be similar to the bacterial examples by measuring PTPP binding.

Table 1. Apparent K_D Values of TPP Riboswitches

B. subtilis	Wild Type	Mutant	Loss of Affinity (Mutant ÷ Wild Type)
TPP	50 nM	1.8 μM	36
PTPP	160 nM	1.6 μM	10
Thiamine	50 μM	nd	nd
PT	6 μM	nd	nd

A. oryzae	Wild Type	Mutant	Loss of Affinity (Mutant ÷ Wild Type)	Consensus Aptamer
TPP	56 nM	4 μM	71	20 nM
PTPP	50 nM	2 μM	40	56 nM

Values were determined in vitro by in-line probing of the constructs detailed in Figures 2 and 3. nd, not determined.

PTPP Binds a Fungal TPP Riboswitch

There is a PT-resistant strain of *A. oryzae* that has been developed and commercialized into a dominant selectable marker for fungal transformations (TaKaRa Bio, Shiga, Japan) [25, 45]. The TPP riboswitch is currently the only riboswitch known in eukaryotes [46] and the mutant fungal allele was found to have a single A to G change in the 5′-UTR with no changes at all in the protein coding region (Figure 3A) [25]. The 5′-UTR of *A. oryzae thiA* was previously shown to contain a consensus TPP riboswitch motif [46]. The sequence from −233 to −61 relative to the translation start site of wild-type *thiA* was tested by in-line probing to confirm it as a TPP aptamer (Figure 3). Distinct changes in the pattern of spontaneous RNA degradation occur in the *A. oryzae* riboswitch at positions G141 and A123. The apparent K_D of this RNA for TPP is 56 nM (Table 1). PTPP is bound by the riboswitch with a nearly identical K_D of 50 nM. These values are also in good agreement with the ligand affinity of a TPP riboswitch from *Arabidopsis thaliana* [46].

The mutation in the PT-resistant allele occurs at a nucleotide that is conserved in >90% of TPP riboswitches examined from bacteria and eukaryotes [40, 46]. The affinity of the mutant riboswitch from the PT-resistant *A. oryzae* strain is reduced by 71-fold for TPP and by 40-fold for PTPP. We also noted that the *thiA* TPP riboswitch carries what appeared to be an A insertion (Figure 3A, nonconsensus) relative to the consensus TPP aptamer (Figure 2B). To test whether this difference alters TPP binding, the construct without this A was prepared and tested by in-line probing. Aptamers with and without this A both bind TPP and PTPP well (Table 1), suggesting that this nucleotide does not influence ligand binding.

These results show that TPP riboswitches from multiple organisms and two domains of life bind PTPP extremely well in vitro and that PT resistance correlates with mutations in TPP riboswitches among evolutionarily diverse organisms. To show directly that PT influences gene expression via TPP riboswitches, we next investigated the expression of riboswitch-reporter gene fusions in transgenic bacteria.

PT Inhibits Expression of Reporter Genes Driven by TPP Riboswitches

Previously, we showed that extracellular thiamine reduces expression of a reporter gene under riboswitch control in *E. coli* [6]. Similarly, it has also been shown that extracellular PT reduces expression of chromosomal *thiA* in *A. oryzae* [25]. In this study, we used reporter gene constructs fused to the TPP riboswitch, without any other chromosomal elements. We expected that PT would enter cells, be pyrophosphorylated enzymatically to PTPP, bind the TPP riboswitch controlling reporter gene expression with high affinity, and thereby repress reporter gene expression. Specifically, riboswitch-reporter gene constructs were created by fusing the appropriate 5′-UTR sequence with a *lacZ* gene. In *B. subtilis* strain 1A40 (PT-sensitive), cells grown in minimal medium produced a Miller value of 66.8 units (see Experimental Procedures) for *lacZ* expression (Figure 4A). When either thiamine or PT was added to the medium, the expression of *lacZ* dropped by more than 5-fold. The mutant TPP riboswitch from strain 1A481 exhibits β-galactosidase activity similar to that of wild type (94 Miller units in medium alone). However, adding either thiamine or PT produced very little or no repression of *lacZ*, showing that the mutant TPP riboswitch fails to regulate genes, as expected.

As a control, the impact of PT toxicity on the reporter assay was assessed by repeating the assay in a strain of bacteria that is resistant to PT. The basal expression of the reporter gene with the wild-type riboswitch is 44.5 Miller units. Because reporter gene expression is not substantially repressed, compared to its expression in the wild-type genetic background, TPP must not be greatly overexpressed in this resistant strain during the incubation time for the assay. All observed results follow the same trend in this background as in the sensitive strain (Figure 4B). Thus, the addition of PT is sufficient to cause repression of genes controlled by TPP riboswitches. Furthermore, mutant riboswitches that cannot bind TPP or PTPP in vitro do not exhibit regulation of reporter gene expression by thiamine or PT.

Reporter assays were also conducted in a wild-type *E. coli* (*lacZ*$^-$) strain transformed with either a wild-type *thiC* TPP riboswitch-gene fusion or with *lacZ* fused to a mutant riboswitch from the PT-R1 strain. The pattern in gene expression is the same as that described for *B. subtilis* (Figure 4C). Thus, multiple examples of bacterial TPP riboswitches regulate genes in response to extracellular PT as though thiamine were being added instead. This indicates that PT enters cells and interacts with TPP riboswitches broadly, most likely in the form of PTPP.

TPP Riboswitches Are Often Mutated in PT-Resistant Bacteria

We hypothesize that a key mechanism of PT toxicity is the binding of PTPP to TPP riboswitches, repression of TPP transport and biosynthetic genes, and starvation of the cell for TPP. In order to provide direct evidence of this model, we sought to analyze additional PT-resistant strains. PT-resistant bacteria are not commonly used in research and the strains that yielded the mutant TPP riboswitches used in these reporter gene assays were the only such examples available in the *B. subtilis* and *E. coli* stock centers. To study PT toxicity more generally, we generated several additional PT-resistant strains. PT-sensitive strains of both *B. subtilis* and *E. coli* were exposed to PT in defined media without thiamine and allowed to incubate until dense growth occurred. Surviving cells were passaged into fresh medium with and without PT until cultures grew identically regardless of PT.

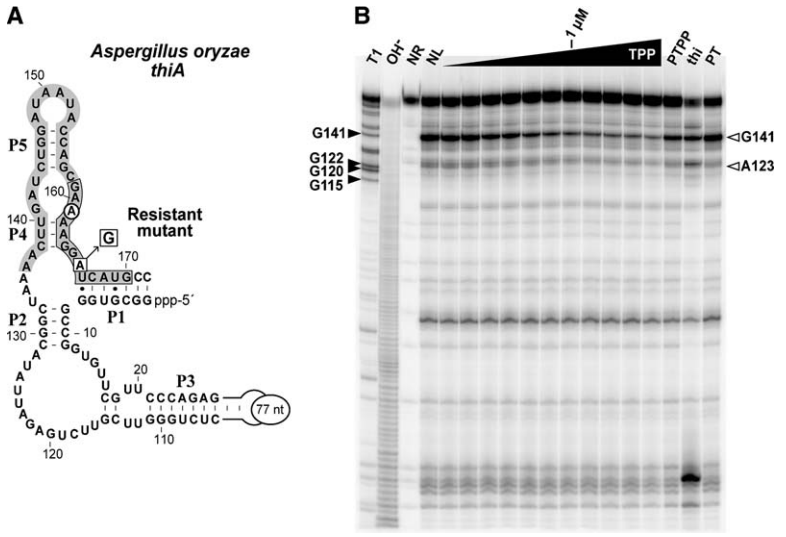

Figure 3. *A. oryzae thiA* TPP Riboswitch

(A) The *A. oryzae thiA* 5′-UTR constructs used for in-line probing. Depicted are nucleotides −233 to −61 of the wild-type 5′-UTR. The boxed G identifies the mutation carried in the PT-resistant allele *ptrA* [25]; large deletions that were previously reported to interfere with regulation and expression are shaded light gray or shaded and boxed. The nonconsensus A residue is circled. Stem P3 is longer than in bacteria, carries occasional mismatches, and has no changes in the mutant.

(B) In-line probing of the wild-type *thiA* riboswitch. RNA was incubated with concentrations of TPP as in Figure 2C, with PTPP at 300 nM, thiamine (thi) at 1 μM, or PT at 1 μM. Other notations are as in Figure 2.

B. subtilis tenA or *E. coli thiC* TPP riboswitches were then sequenced from individual colonies to check for mutations in regulation of the major TPP biosynthetic operons.

Surprisingly, all of the PT-resistant *B. subtilis* clones acquired mutations in the *tenA* riboswitch (Figure 5A). These are mostly point mutations that alter conserved nucleotides in the TPP aptamer (Figure 5A, compare to Figure 2B) but one clone carries an insertion in the expression platform also. The preponderance of *tenA* riboswitch mutations in PT-resistant *B. subtilis* suggests that this riboswitch is the primary cellular PT target.

Interestingly, the sequencing of the *E. coli* mutants yielded a different pattern of mutations. Only 7 of the 23 clones carry mutations in the *thiC* riboswitch (Figure 5B). The remaining 16 *E. coli* clones were then sequenced at the other two TPP riboswitches. One carried a U insertion in the conserved P2 stem of the *thiM-thiD* operon riboswitch, which is expected to greatly weaken this stem and prevent TPP/PTPP binding (data not shown). Because the phosphorylation of HMP-P catalyzed by *thiD* is essential for thiamine biosynthesis, it is possible that derepression of the gene maximizes conversion of HMP-P to TP and confers PT resistance. Another clone carries a G insertion in the nonconserved loop of P3 of the riboswitch controlling the *tbpA-yabK-yabJ* operon, which is not expected to impact ligand binding and is therefore unlikely to be functionally relevant. Mutations in the *tbpA* riboswitch that prevent PTPP binding and derepress these genes would be expected to increase toxicity in defined (thiamine-free) medium, as these thi/TP/TPP transporters likely transport PT into the cell. Presumably, the remaining 14 clones from *E. coli* that do not carry riboswitch mutations have gained resistance by some other mechanism.

B. subtilis May Hydrolyze PT to Escape Toxicity

The functions of all of the *E. coli* genes regulated by TPP riboswitches are known to be involved in thiamine biosynthesis and transport. Analogous genes are known to be controlled by TPP riboswitches in *B. subtilis* as well. However, a few *B. subtilis* genes without a known

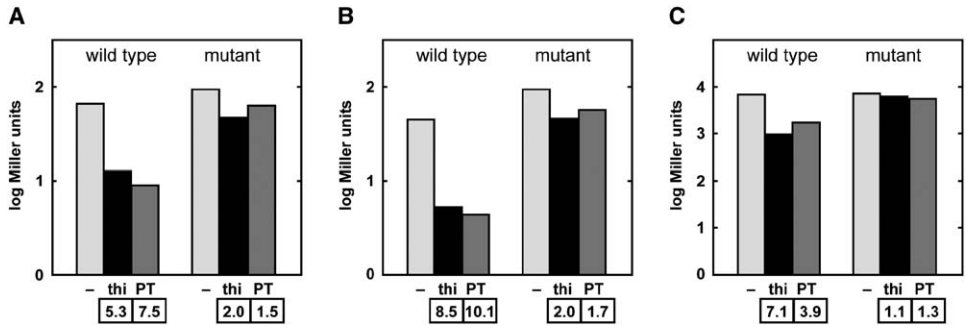

Figure 4. Riboswitch Regulation of *lacZ* Expression in Response to PT

(A) β-galactosidase expression controlled by a TPP riboswitch in wild-type *B. subtilis*. Cells were incubated in defined media with no added supplement (–), with 10 μM thiamine (thi), or with 10 μM PT (PT). *B. subtilis* has no endogenous β-galactosidase activity, and all expression is due to the reporter gene. The riboswitch used to control the *lacZ* gene is indicated above each cluster of results: either PT-susceptible (wild-type) or PT-resistant (mutant). The amount of repression of β-galactosidase activity measured in Miller units is shown boxed as the ratio without supplement and with either thiamine or PT.

(B) Same assay as in (A) but in PT-resistant strain 1A481.

(C) Reporter assay with *E. coli*. The assay was performed as in (A) with an *E. coli* strain that is PT sensitive and *lacZ*$^{-}$. The PT-resistant strain is *lacZ*$^{+}$ and so could not be used as a control for PT toxicity as in *B. subtilis*.

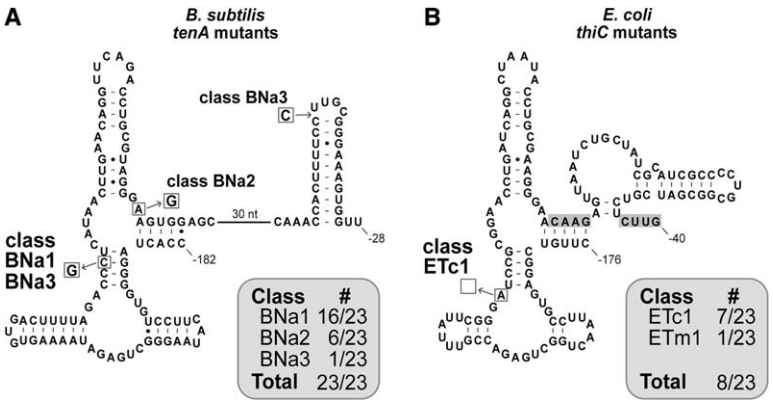

Figure 5. Riboswitch Mutations Conferring PT Resistance

(A) All 23 of the *B. subtilis* clones bore mutations in *tenA* TPP riboswitches and were distributed into three classes termed BNa1, BNa2, and BNa3.

(B) In *E. coli*, seven clones acquired mutations in *thiC* TPP riboswitches (class ETc1) and one clone acquired a mutation in the *thiM* riboswitch (class ETm1, not shown). The expression platform of the *E. coli* riboswitch is a Shine-Dalgarno hiding mechanism (shaded). The remaining 15 clones do not have mutations in TPP riboswitches.

role in thiamine metabolism (*tenA*, *tenI*, and *ylmB*) are regulated by TPP riboswitches. TenA and TenI were originally reported to be transcriptional enhancers when overexpressed [47]. Bioinformatics has revealed, though, that *tenA* is associated with thiamine metabolism genes and TPP riboswitches in most bacteria [39]. We have already shown that although *B. subtilis* has a complete TPP biosynthetic pathway that could be targeted by PT at both the RNA and protein level, mutations are always observed in the *tenA* riboswitch (which regulates both *tenA* and *tenI*) in PT-resistant clones. Recent structural and biochemical analysis of TenA from *B. subtilis* reveals it to be a type II thiaminase [48, 49]. Other recent structures of archeal TenA homologs support different conclusions [50, 51], though these lack biochemical supporting data and may not be relevant to eubacterial TenA. The physiological roles of either type I or type II thiaminases are currently unknown [52–54]. However, the regulation of *tenA* expression by TPP riboswitches, the assignment to TenA of a function related to thiamine metabolism, and the targeting of the *tenA* riboswitch specifically by PT all support a role for TenA in thiamine metabolism in *B. subtilis*.

We suggest a model that assigns a role for TenA both in normal thiamine metabolism and PT resistance. All of our PT-resistant *B. subtilis* clones are predicted to carry *tenA* riboswitches with mutations that prevent binding of either TPP or PTPP and therefore prevent repression of the genes in the *tenA* operon. If so, then these strains constitutively express a thiaminase despite, as we propose, being subjected to PT-induced thiamine starvation. It is possible that TenA accepts PT as a substrate, directly detoxifies it by hydrolysis, and contributes HMP to the salvage pathway for thiamine biosynthesis (Figure 6). Type II thiaminases hydrolyze thiamine into HMP and Thz but do not act on TPP [55]. Type I thiaminases accept a variety of nucleophiles and catalyze an exchange with either thiamine or TPP that yields free Thz (or Thz-PP) and an adduct of HMP. TenA was shown to have an affinity for HMP [48], which is the common moiety between thiamine and PT. The model type I and type II thiaminases from *Bacillus thiaminolyticus* (*Paenibacillus thiaminolyticus*) and *Bacillus aneurinolyticus*, respectively, have been reported to have activity against a pyrimidinyl-pyridine compound (of which PT is a type) [52]. Furthermore, the effects of PT were measured on fungal species, including two that cannot biosynthesize HMP and require HMP supplementation. These fungi grow if provided with subtoxic amounts of PT, presumably by obtaining HMP from PT and condensing it with biosynthesized Thz-P [11]. This suggests the presence of an enzyme that recovers HMP from PT in these fungi.

In our model, HMP liberated from PT by TenA would be phosphorylated to HMP-PP by YjbV, and could be a source of HMP-PP despite continued repression of *thiC* by PTPP binding to its riboswitch. Therefore, all of the genes necessary to generate HMP-PP and Thz-P are derepressed in a strain with a mutated *tenA* riboswitch. ThiE coupling will proceed normally. Any free thiamine could be degraded by the elevated TenA concentration; however, the HMP and Thz moieties would simply be rephosphorylated, recoupled to TP, and rephosphorylated to TPP. Degradation of TPP by TenA would not occur because TenA does not act on TPP [48].

This model requires that type II thiaminases primarily recognize thiamine via the HMP moiety rather than the entire molecule. It has been suggested before that HMP serves as a recognition site for a thiamine transporter that also transports the thiamine analog amprolium [56], and PT could therefore enjoy a similar level of recognition by TenA. Furthermore, the role of TenA as a thiaminase is consistent with a previous report that *B. subtilis* TenA complements a *thiC* deletion in *E. coli* [57]. The *E. coli* cells could compensate for an inability to synthesize HMP via ThiC by increasing their salvage of HMP via TenA from PT or other HMP-containing compounds such as those produced by the activity of type I thiaminases. Overall, this model provides one possible explanation of the marked bias observed in the type of mutations correlating with PT resistance between *B. subtilis* and *E. coli*. It serves to explain this bias by concentrating on the difference in the genes encoded by the major TPP biosynthetic operons between these two organisms, namely the *tenA* and *tenI* genes. However, there are certainly alternative explanations and further testing of TenA function is needed to distinguish among these possibilities.

As a result of this study on the interactions between PT and TPP riboswitches, two new hypotheses emerge. The first is that the physiological role of TenA is to hydrolyze HMP-containing compounds, such as those generated by type I thiaminases. The second is that TenA directly detoxifies PT and that derepressing its synthesis by mutation to its TPP riboswitch is an effective strategy for resisting PT toxicity. The binding and reporter

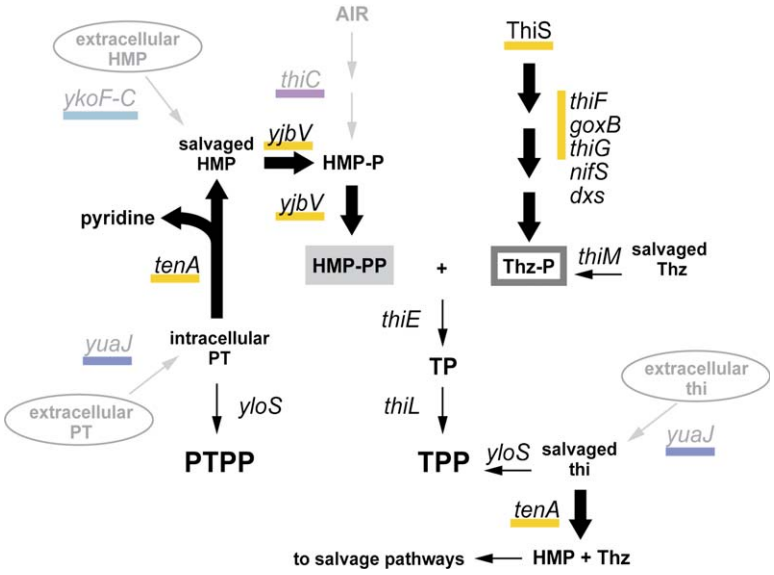

Figure 6. Model of *B. subtilis* PT Resistance

The effect of derepression of the *tenA* operon on gene expression in PT-resistant mutants is shown. Components of the pathways in gray type remain repressed. Thick black arrows indicate derepression and thin black arrows denote no riboswitch-mediated change in expression. The putative detoxifying role in PT resistance of TenA as a type II thiaminase and pyrithiaminase is shown.

expression data contained in this report validate TPP riboswitches as antimicrobial drug targets. These results indicate that both TPP and lysine riboswitches are targets of antimicrobial drugs. Both PT and AEC were first synthesized several decades ago but the full impact of their physiologic effects has remained unknown. The demonstration now that both compounds interact with riboswitches indicates that additional metabolite analogs could be actively developed for riboswitch binding, leading to antimicrobial effects.

Significance

In this report, we have demonstrated that PT exerts toxic effects by interacting with TPP riboswitches. Reporter protein expression regulated by wild-type TPP riboswitches is influenced both by extracellular thiamine and PT. Furthermore, bacterial and fungal populations can both acquire PT resistance by selecting mutations within specific TPP riboswitches. In vitro affinity for both TPP and PTPP is reduced in these mutant riboswitches and riboswitch-reporter gene expression is no longer regulated in response to extracellular PT or thiamine. These results suggest that PT is imported, phosphorylated to the biologically active form PTPP, bound as PTPP to TPP riboswitches with high affinity, and responsible for repressing expression of thiamine biosynthesis and import genes. PT could then exert toxic effects by starving cells for TPP, although other effects of PT on proteins might also occur. PT resistance can be attained by mutating TPP riboswitches to abolish ligand binding and converting expression of the TPP-related genes to constitutively high levels. Patterns of PT resistance can both help further define thiamine metabolic pathways in bacteria and also guide future drug development.

Our findings provide validation that TPP riboswitches are antimicrobial drug targets. This indicates that antimicrobial compounds known for decades function at least in part by a riboswitch-related mechanism. This also suggests a general antimicrobial drug development approach of generating analogs of riboswitch ligands that bind to riboswitches and disrupt regulation of key metabolic pathways. Riboswitches are attractive as targets for new antimicrobial drugs. Riboswitches naturally evolved to bind "drug-like" molecules as small as 100 Da, and it is expected that this natural binding ability will offer extensive opportunities for generating and screening small molecule riboswitch inhibitors. Also, riboswitches control gene expression associated with the levels of coenzymes and other critical metabolites whose proper regulation is expected to be essential for optimal growth.

Experimental Procedures

RNA Preparation and In-Line Probing

Chemicals and oligonucleotides were purchased and prepared as reported previously [6]. RNA was transcribed in vitro from synthetic DNA templates by T7 RNA polymerase. RNAs were prepared and labeled at the 5′ end with ^{32}P as described previously [58]. For in-line probing, RNA was incubated for ~40 hr at 25°C in 20 mM $MgCl_2$, 100 mM KCl, 50 mM Tris·HCl (pH 8.3) at 25°C in the absence or presence of various concentrations of thiamine, TPP, PT, or PTPP. Under these conditions, spontaneous RNA breakdown due to positioning effects is high enough to be measured accurately and reproducibly but slow enough to limit degradation to one or a few events per RNA molecule. RNAs are separated by denaturing PAGE on high-resolution gels.

PTPP Synthesis

Pyrithiamine pyrophosphate was prepared in the same manner as hydroxyethylthiamine pyrophosphate syntheses reported previously [59, 60]. Pyrithiamine hydrobromide (fw 420.1) and phosphoric acid crystal (99.999+%) were obtained from Sigma Aldrich (Milwaukee, WI). Crystalline phosphoric acid (400 mg, 4.08 mmol) was heated over flame in a bottle with a loosely fitted aluminum foil cover. The crystals liquefied, bubbled slightly, and then turned cloudy. This liquid was cooled to room temperature. Pyrithiamine hydrobromide (7.5 mg, 17.9 μmol) was added to the bottle along with a stir bar (3–5 mm). The bottle was covered loosely with aluminum foil and heated in an oil bath at 105°C–110°C with gentle stirring for 15 min. This mixture was cooled to room temperature. To quench the reaction (~2 ml of 2.04 M phosphoric acid and 8.9 mM pyrithiamine derivatives), 1.6 ml of D_2O was added. The bottle was tightly capped and the mixture vortexed to dissolve the contents in D_2O (white solid remained undissolved). The clear solution was transferred to polyethylene

tubes. After centrifugation, 0.8 ml of the uppermost solution was transferred to an NMR sample tube and analyzed by ^{1}H NMR and ^{31}P NMR on Bruker (Fällanden, Switzerland) 400 MHz or 500 MHz NMR spectrometers. The proton NMR spectrum (internal reference: D_2O with 2.04 M phosphoric acid) was almost identical to that of pyrithiamine except for protons close to phosphate ester: pyrithiamine (14.8 mM in D_2O with 2.04 M phosphoric acid) δ 3.00 (2H, t, J = 6.20 Hz), δ 2.24 (2H, t, J = 6.40 Hz); pyrithiamine mono-, di-, triphosphate (14.8 mM in D_2O with 2.04 M phosphoric acid) δ 3.51 (2H, b), δ 2.53 (2H, b). The proton-decoupled phosphorus NMR spectrum showed three peaks (chemical shifts relative to 2.04 M phosphoric acid) δ –0.55 (monophosphate), δ –11.4 (pyrophosphate), δ –24.5 (triphosphate). The conversion of the reaction determined by proton NMR was >98% and the ratio of the phosphate esters determined by phosphorus NMR was <1% monophosphate, 85% pyrophosphate, and 15% triphosphate. This 7.57 mM pyrithiamine pyrophosphate solution (85% of 8.9 mM) contained no detectable side products by NMR and could be used for the biological experiments simply after dilution with buffer to adjust pH. In addition, PTPP was prepared for related experiments and purified by reverse-phase HPLC. A *B. subtilis* TPP riboswitch tested with the purified PTPP was found to have approximately the same dissociation constant (± 2-fold) with the purified PTPP as with PTPP prior to HPLC separation (data not shown).

Quantitation of Apparent Dissociation Constants

In-line probing gels were used to determine apparent K_D values for both the wild-type and mutant RNA sequences. Apparent K_D values were determined by quantitating (ImageQuaNT; GE Healthcare/Amersham Biosciences AB, Piscataway, NJ) the amount of RNA degradation for a given nucleotide position over a range of ligand concentrations. The amount of degradation was normalized from 0 (no change in RNA degradation with and without ligand) to 1 (maximum change in degradation with and without ligand) for each site. The apparent K_D was determined by visual inspection of the plot of normalized degradation (fraction cleaved) vs. ligand concentration as demonstrated previously [6].

Construction of TPP Riboswitch-*lacZ* Fusions

Fusion of the *tenA* riboswitch with a *lacZ* reporter gene was used to assess the expression in *B. subtilis* using methods similar to that described previously [28]. The *tenA* DNA region comprising nucleotides −261 to +11 with respect to the translation start site was PCR amplified as an EcoRI and BamHI fragment from the PT-resistant strain 1A481 (*Bacillus* Genetic Stock Center, The Ohio State University, deposited by A. Gelizze) This sequence carries the point mutation G-94 to A on the riboswitch, relative to the translation start site. The PCR-amplified DNA was cloned into the pDG1661 reporter vector lacking an independent translation start site. A wild-type variant of this construct was generated by oligonucleotide-directed mutagenesis using wild-type primers. Constructs were integrated in the *amyE* locus in strain 1A40 and confirmed as described [32]. The *E. coli thiC-lacZ* fusion was constructed previously [6].

Thiamine Repression of β-Galactosidase Assays

Cells were grown as described [28] in defined medium under routine conditions to an A600 of 0.2, at which time cells were split into three aliquots in defined medium with and without supplements as noted. Cultures were incubated for an additional 3 hr before performing the β-galactosidase assay, similar to that of Miller [61].

Evolution of PT-Resistant Mutants

Resistant mutants were acquired with a method similar to a published report [44]. A fresh overnight culture was diluted 1/100 in minimal medium with and without 100 μM PT. For *E. coli*, these cultures were grown at 37ºC protected from light without shaking. *B. subtilis* cultures were grown at 37ºC protected from light with shaking. When PT-containing cultures were saturated, 10 μl was used to inoculate 1 ml each with minimal medium with and without 100 μM PT. Passages were continued in this way until the culture with PT became saturated in approximately the same time as those without PT (five passages).

Sequencing

The 5′-UTR of the *thiC* operon from *E. coli* strain PT-R1 was sequenced with primers EcThiC1 and EcThiC2 to amplify −258 to +10 relative to the translation start site. Twenty-four additional PT-resistant *E. coli* clones were sequenced with these primers as well. Twenty-three reactions gave readable sequences. The 16 PT-resistant *E. coli* clones that did not have mutations in the *thiC* TPP riboswitch were then sequenced in the 5′-UTR of both the *tbpA* and *thiM* operons. Sequencing of *tbpA* 5′-UTR was done with primers EcTbpA1 and EcTbpA2 (−226 to −1, relative to translation start), sequencing of *thiM* 5′-UTR with primers EcThiM1 and EcThiM2 (−172 to +2).

EcThiC1: 5′-TAAGCGAATTCTGCTGGTGCTTGACG-3′
EcThiC2: 5′-CGCGTAGATCTCGGCGGGTCAGTTTTG-3′
EcTbpA1: 5′-GGGAATTCGCAACGCAGTATGCGCGGCCTG-3′
EcTbpA2: 5′-GACGGGATCCCCCAGCAGGGGCAGACATTTTTTTAACAC-3′
EcThiM1: 5′-CTGCCGGAATTCCTCGTTTACAACGCGTG-3′
EcThiM2: 5′-TTGCGCTGGATCCAGCAGGTCGACTTGC-3′

Supplemental Data

Supplemental Data include one figure and one table and can be found with this article online at http://www.chembiol.com/cgi/content/full/12/12/1325/DC1/.

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